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## ABSTRACT

Message levels of premalignant (S2) and tumorigenic (T4-2) cells, two sublines of a human breast cancer series, were compared to identify molecules marking the final events in malignant transformation. A novel gene AZ-1 expressed at a >10-fold higher level in S2 than in T4-2 cells was isolated. While AZ-1 message was present in nonmalignant breast cell lines, primary luminal epithelial cells, and cells from reduction mammoplasty, it was drastically downregulated or absent in ten mammary carcinoma cell lines and four breast carcinoma biopsies. Ectopically-expressed AZ-1 in T4-2 cells reverted them to a normal-like phenotype in a three-dimensional basement membrane culture. Interestingly, upregulation of AZ-1 message was also correlated with phenotypic normalization of breast tumor cells in a  $\beta$ 1-integrin reversion system. Furthermore, AZ-1 stably-transfected T4-2 cells were 80% less invasive and clonogenic in matrigel invasion and colony-forming assays, respectively, than the vector transfectants. Second structure predictions indicated that C-terminus of AZ-1 is homologous to the rod domain of plakin family, the versatile organizers of cytoskeletal architecture. These data suggest that AZ-1 may participate in the cytoskeletal organization of breast epithelial cells and AZ-1 may provide a link to the understanding of the abnormal cell-cell and cell-extracellular matrix interactions in the breast tumor cells.

## INTRODUCTION

Human breast cancer is thought to derive from the stepwise transformation of the luminal epithelial cells of the ducts and terminal lobular units (1,2). This has led to the conclusion that mutational events are critical to the genesis of the transformed phenotype. Mutations studied include the amplification or aberrant expression of the proto-oncogenes: *c-myc*, *c-erbB2*, *int-2/hst-1* and infrequently *H-ras*, as well as, inactivation or deletion of tumor suppressor genes such as *p53*, *Rb-1*, and *BRCA1* (3). Despite these clinical correlations, at present it is not yet possible to ascribe a pivotal role for a specific genetic mutation in breast cancer aetiology. To clarify this issue, it would be desirable to follow the progressive changes until malignancy ensue in the tissue *in vivo*. However, the time-course of breast tumor evolution can be quite long, taking anywhere from 5-30 years to develop. To follow the development of carcinoma *in situ* to invasive carcinoma, epidemiological protocols must span periods of 10-15 years (4). This precludes the feasibility of conducting meaningful and reproducible human studies and emphasizes the need to develop appropriate experimental models of human breast cancer progression in culture.

It is now widely accepted that extracellular matrix (ECM) is a key component of tissue microenvironment playing a determinant role in functional differentiation of developing and adult epithelia (5-7). In the mammary gland, extensive data exist showing that basement membrane components regulate the morphological and functional differentiation of mammary epithelial cells in culture and *in vivo* (8-10). In addition, altered interactions with ECM have been observed in mammary tumor development, emphasizing the importance of microenvironmental regulation in normal development and malignancy (11-13). Signals provided to mammary epithelium by basement membrane may be mediated by integrins, the transmembrane heterodimeric cell-surface receptors that link ECM to structural and functional elements within the cell (14-16). Several

integrin receptors for laminin, the main component of basement membrane including  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  are expressed in normal human mammary epithelium. Altered expression of these receptors is a common occurrence in breast tumors (17-19). The disrupted tissue architecture observed in mammary adenocarcinoma is also frequently associated with alterations in integrin heterodimer profiles (20,21). Changes in  $\beta 1$ -,  $\beta 4$ -,  $\alpha 2$ -,  $\alpha 3$ - and  $\alpha 6$ -integrins have been reported for mammary tumor cell lines and in tissue sections, and were shown to be associated with tissue disorganization, loss of polarity, increased tumor aggressiveness, and metastasis (22). Integrins are known to possess intrinsic kinase activity and to require associated molecules for signaling. The emerging concept is that integrin cooperatively works with linked kinase, or growth factor receptors, or also through their interactions with the cytoskeletal components. However, a relationship between altered signal transduction via integrins and the adherens junction pathways, and its relevance to the origin of the tumor phenotype has not been directly examined. This is mainly due to the lack of appropriate model systems in which such changes can be studied (23).

We have thus taken advantage of a unique epithelial cell model of breast cancer developed by Briand and coworkers (24,25). The HMT-3522 breast cancer series was established under chemically defined conditions from a breast biopsy of a woman with a nonmalignant breast lesion (26). The established cell line S-1 is entirely dependent on exogenous epidermal growth factor (EGF). In passage 118, cells were adapted to grow in medium without EGF and a new growth-transformed subline S-2 cells was generated and propagated at a high growth rate without exogenous EGF (27). A highly dramatic shift in phenotype was observed in passage 238 when the S-2 cells became tumorigenic in nude mice. After two mouse-culture passages, the resulting malignant transformed cell line (T4-2) was refractory to the growth-modulating effect of EGF and presented an extra copy of a chromosome marker, 7q- (25). These three cell lines (S-1, nonmalignant, S-2, premalignant, and T4-2, tumor) one originating from the other by spontaneous genetic events, therefore, provide a unique tool for addressing the carcinogenic event and particularly for us the altered ECM-signaling pathways through integrins involved in malignant conversion in the breast.

In addition to elucidating integrin signaling, we have also exploited molecular approach to search for candidate genes that might be involved in cellular transformation. Differential display originally described by Liang and Pardee (28) offers a powerful tool for this endeavor. The method is based upon comparison of mRNAs expressed in two or more cell populations by running their reverse transcribed and radioactively-labeled PCR products on sequencing gels in adjacent lanes. The bands revealing detectable differences between cell populations are cut and the cDNAs are eluted. After PCR re-amplification, the eluted cDNAs can be directly used as probes in northern blots for verification and in subsequent recovery of the full-length clones from cDNA libraries. By using suitably chosen PCR primers, the majority of the cDNAs can be displayed as 100-600 bp fragments. Several candidate tumor suppressor genes in breast cancer including  $\alpha 6$  integrin were identified by this method (29).

Using this differential display approach, we have isolated a novel tumor suppressor candidate gene AZ-1. Sequence analyses and secondary structure predictions indicated that C-terminal coiled-coil domain of AZ-1 protein showed sequence homology to the rod domain of plakin family proteins.

Plakin family comprises four sequence-related proteins: desmoplakin, plectin, bullous pemphigoid antigen 1 (BPAG1) and envoplakin (30). While desmoplakin and BPAG1 are constituents of desmosome and hemidesomes respectively, plectin is expressed more widely. They are mainly localized to intermediate filaments (IF) and filament attachment sites at the plasma membrane and they are recognized as versatile organizers of cytoskeletal architecture. The plakins are predicted to contain globular amino- and carboxyl-terminal domains that are separated by a central coiled-coil rod domain (31-36). The central rod domain is rich in heptad repeats and is believed to form a parallel  $\alpha$ -helical coiled-coil with a dimerization partner or to assemble into higher-order filamentous structures with themselves or other coiled-coil proteins (30). Loss of plectin function, as in the case with BPAG1, has been linked to skin diseases such as epidermolysis bullosa possibly caused by disruption of anchorage site of keratin filaments to hemidesmosomes (37). On the other hand, abnormal expression of desmosomal proteins such as desmoplakin contributed to tumorigenicity and invasion in bladder and other cancers (38-40).

## RESULTS AND EXPERIMENTAL METHODS

*Specific aim 1: Define the ECM ligands that elicit normal breast and breast tumor phenotypes in a reconstituted basement membrane.*

Murine mammary epithelial cell lines competent for milk protein expression were used to identify microenvironmental factors that regulate lactoferrin, a secreted iron binding protein which is expressed during normal functional development of mammary epithelium.

(A) Lactoferrin expression in mammary epithelial cells is mediated by changes in cell shape and actin cytoskeleton (see publication #1)

### **Induction of lactoferrin gene expression in cultured mouse mammary epithelial cells by laminin**

Tissue-specific expression of milk protein genes in primary and secondary mouse mammary epithelial cells has previously been shown to depend on lactogenic hormones and contact with reconstituted basement membrane. To begin to determine the specific factors which influence lactoferrin (LTF) gene expression in mammary epithelium, the CID-9 mouse mammary epithelial cell line, was cultured in the absence or presence of reconstituted basement membrane (EHS matrix) with or without the mammotrophic hormones, prolactin. LTF message was not observed in total RNA harvested from medium density culture of CID-9 attached to tissue culture plastic, but was easily demonstrated in RNA from the same cells attached to EHS matrix. Unlike the caseins, the expression of LTF was independent of prolactin. Further characterization of the matrix effect was performed with a clonal derivative of CID-9 cells, SCp2. Growing cultures of SCp2 cells, initially negative for LTF mRNA expression were deprived of fetal calf serum for 72 hours, then given 50  $\mu$ g/ml of purified laminin, a major basement membrane component, or 5% fetal calf serum in the culture medium for an additional 6 or 48 hours prior to harvest. The cells exposed to laminin aggregated into clusters or round, refractile cells. In the culture exposed to fetal calf serum, the

SCp2 cells achieved confluence by 48 hours. Incubation with either laminin or fetal calf serum was sufficient to induce LTF mRNA expression by 48 hours. This result was in striking contrast to  $\beta$ -casein mRNA which specifically required laminin for expression. The fetal calf serum result suggested that changes in cell-cell interactions, cell growth, and/or cell shape could replace the conditions for LTF induction provided by basement membrane.

*Specific aim 2: Identify the specific integrins (ECM receptors) that transduce the ECM signal to the cells, by examining their expression in normal and tumor cells and elucidating their functional role by disrupting the signaling pathways.*

(A) Cellular growth and survival are mediated by  $\beta 1$  integrins in normal human breast epithelium but not in breast carcinoma (see publication #2)

### **Formation of acinar structures within EHS is integrin dependent**

To determine which of the various integrins expressed by human breast epithelial cells are functionally relevant to the formation of the acinar structures, specific inhibitory anti-integrin antibodies were used to interfere with this process in reconstituted basement membrane culture. Nonmalignant S1 cells cultured in EHS without antibodies, or with 10  $\mu\text{g/ml}$  or 100  $\mu\text{g/ml}$  of non-immune mouse or rat IgG, formed well-organized acinar structures at similar frequencies. In contrast, inhibitory anti- $\beta 1$  subunit antibodies, at similar concentration, severely impaired the formation of spheres by S1 cells relative to control cultures. These effects were observed with two different anti- $\beta 1$  antibodies and were dose dependent: mouse anti- $\beta 1$  mAb (JB1a) induced a two-fold inhibition of sphere formation at 40  $\mu\text{g/ml}$  and a 4-fold inhibition at 200  $\mu\text{g/ml}$ , whereas rat anti- $\beta 1$  mAb (AIIB2) caused an almost complete inhibition at 100  $\mu\text{g/ml}$ . These data suggested that sphere formation by normal human mammary epithelial cells in response to EHS is dependent on integrin(s) of the  $\beta 1$  integrin family.

To define more precisely which integrins were critical in signaling acinar morphogenesis, similar experiments were performed with inhibitory anti- $\alpha 2$ , - $\alpha 3$ , and - $\alpha 6$  antibodies. Anti- $\alpha 3$  reduced sphere formation by 50% at 10  $\mu\text{g/ml}$  and by 80% at 100  $\mu\text{g/ml}$ . Anti- $\alpha 5$  and anti- $\alpha 6$  antibodies were much less effective (about 70% of control) at both 10  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$ , while inhibitory anti- $\alpha 2$  antibodies showed no appreciable inhibition. These data indicate that, of the integrin subunits assayed,  $\alpha 3\beta 1$  appears to be the most significant in mediating morphogenesis in a basement membrane matrix.

### **The inhibition of acinar morphogenesis by anti-integrin antibodies is associated with an inhibition of cell growth**

Since normal mammary cells seeded into EHS in the presence of inhibitory anti-integrin antibodies remain suspended as single cells for the duration of the experiments, we asked whether the inhibitory antibodies interfered with acinar formation by blocking cellular growth. The thymidine-labeling indices (TLI) of S1 cells cultured in EHS in the presence or absence of inhibitory anti-integrin antibodies were measured. In the absence of inhibitory anti- $\beta 1$  antibodies the TLIs were

approximately 60% at day 2 and fell to 3% in EHS by day 6 as the cells formed differentiated acini. In contrast, in the presence of inhibitory antibodies, the cells remained suspended as single cells, and the TLIs were low at day 2 in both substrata and remained low throughout the experiment. At day 6 of culture in EHS, the low TLIs observed in the presence of anti- $\beta$ 1 antibodies reflect a small number of growth-arrested differentiated cells that formed acini by escaping the antibody blockade, and a majority of growth-inhibited single cells blocked by anti-integrin antibodies. These data suggest that an initial phase of cell growth is a requirement for acinar formation in three-dimensional culture. Thus, acinar formation appears to be a two-step process involving a  $\beta$ 1-integrin-dependent cellular growth phase, followed by a phase of cell polarization to form the final organized structure.

### **Interruption of normal mammary cell-basement membrane interactions induces apoptosis**

Prevention of appropriate cell-ECM contact by use of non-adhesive (polyHEMA) coated substrata, ECM fragments or RGD peptides can inhibit cell growth and differentiation in anchorage-dependent cells (Hayman et al., 1985; Ingber, 1990) and trigger programmed cell death or apoptosis (Frisch and Francis, 1994). Data presented herein show that inhibition of mammary cell attachment to basement membrane by ligation of  $\beta$ 1 integrins blocks cellular growth and acinar formation. We therefore asked whether the inhibition of mammary cell-BM interaction induces apoptosis. S1 cells were embedded within EHS matrix in the presence or absence of function blocking anti- $\beta$ 1 antibodies and assayed for evidence of apoptosis at day 2 and day 6 of culture by detection of FITC-digoxigenin nucleotide labeling of 3'OH DNA ends using the ApopTag in situ apoptosis detection kit.

In the absence of anti- $\beta$ 1 antibodies, S1 cells formed acinar structures as described above. Apoptotic nuclei were detected infrequently (0.74%) at day 2, whereas at day 6, 6.3% of single cells not incorporated into acini and 2.5% of individual cells within acini were stained, with the ApopTag reagents. In contrast, in the presence of anti- $\beta$ 1 antibodies, 20.9% of the cells contained nuclei stained by ApopTag reagents at day 2 and at day 6, 59.9% of the nuclei were labelled. Interestingly, a small number of acini (~6% of control) formed in the presence of inhibitory anti- $\beta$ 1 integrin. The cells within these acini did not contain nuclei stained with the ApopTag reagents. These data suggest strongly that  $\beta$ 1 integrins transmit signals from ECM that are required for survival.

(B) Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies (see publication #4)

### **Function Blocking $\beta$ 1-Integrin Antibodies Cause Dramatic Phenotypic Reversion of the T4-2 cells.**

Since previous results showed that T4-2 cells had both a higher total level and an elevated ratio of cell surface  $\beta$ 1- to  $\beta$ 4-integrins, we wondered whether the aberrant malignant behavior may be a reflection of the changes in these integrins. Accordingly, we examined the consequences of treatment in 3-D with varying concentrations of a previously characterized rat monoclonal  $\beta$ 1-integrin antibody (clone AIIB2) which has been shown to inhibit ligand binding (Werb et al., 1989). The antibody caused massive apoptosis in S1 cells (), while T4-2 cells were refractory. Remarkably however, in

addition to resistance to apoptosis, almost all the antibody-treated T4-2 tumor cells assumed a morphology which was indistinguishable from that observed S-1 cultures and was discernible as early as 4 d after incubation. To determine whether the antibody-treated T4-2 cells have truly reverted to a "nonmalignant" phenotype, we cryosectioned the colonies and examined their morphology by immunofluorescence confocal microscopy. As markers of normal acinar formation, we examined both cytoskeletal organization and superimposition and distribution of cadherins and catenins. Sections of S-1 acini revealed uniform and polarized nuclei (stained with propidium iodide; red), well-organized filamentous actin (FITC phalloidin; green), and uniformly superimposed E-cadherin and  $\beta$ -catenin at the lateral cell-cell junctions. In contrast, untreated or IgG-treated tumor cells had polymorphic nuclei and a grossly disorganized actin cytoskeleton, visualized as random, hatched bundles. Additionally, E-cadherin and  $\beta$ -catenin were not colocalized. In contrast,  $\beta$ 1-treated T4-2 cells revealed striking rearrangements of cytoarchitecture as demonstrated by their well-organized acini, and cytokeratin 18 intermediate filament networks. Furthermore, organized adherens junctions became evident in T4- $\beta$ 1 acini and were accompanied by the re-establishment of E-cadherin-catenin complexes. These changes were shown to occur in greater than 95% of the tumor colonies treated with blocking antibody, as quantified by analyzing the numbers of disorganized vs organized spheroids in relation to the S-1 and the mock-treated T4-2 cells.

To investigate whether the described phenotypic reversion is associated with cell cycle regulation, markers of proliferation and cell cycle status was examined in T4- $\beta$ 1 cells. These cells showed a decrease in [ $^3$ H]thymidine incorporation and the size of the acini which was now composed of only 6-8 cells, similar to that observed for S-1 cells. Cryosections of T4- $\beta$ 1 colonies incubated with antibodies against either collagenIV or laminin revealed deposition of a basally distributed, almost continuous basement membrane, with characteristics similar to that observed in the S-1 acini. In contrast, punctate and inversely polarized collagen IV and laminin immunostaining were observed in the mock-treated tumor colonies. Thus, these tumor cells had retained the ability to deposit a basement membrane and to form polarized structures if the correct structural cues could be received. T4- $\beta$ 1 colonies also had dramatically decreased cyclinD-1 levels again comparable to that seen in S-1 cultures and markedly reduced Ki-67 levels. In addition, T4- $\beta$ 1 cells revealed a drastic increase in the negative regulator of cell cycle p21. From these results, it is suggested that most reverted cells had exited the cell cycle and therefore had a reduced propensity to proliferate.

### **Reduced tumorigenicity of $\beta$ 1-inhibitory antibody treated T4-2 cells in nude mice**

To find out whether phenotypic reversion of tumor cells would be sufficient to reduce tumorigenicity in vivo, we injected tumor cells treated in suspension with  $\beta$ 1-integrin blocking mAb, mock mAb, or no treatment for 3 h, as well as S-1 cells into nude mice. Within two weeks small nodules were observed in all injected sites including the S-1 controls. Whereas these nodules regressed rapidly in the S-1 and T4- $\beta$ 1 groups, actively growing tumors were observed in greater than 75-90% of the mock mAb or vehicle-treated T4-2 mice. Upon sacrifice we observed both a significantly reduced tumor number and tumor size in the T4- $\beta$ 1 group. These data suggested that "normalization" of the tumor cell phenotype in culture has a counterpart in vivo where the malignant potential is reduced or lost.

*Specific aim 3: Study the potential role of tumor suppressor genes in the regulation of breast cell-*

*ECM interaction by transfecting these genes into breast tumor cells and assessing the consequence of expression on tumor cell growth and differentiation.*

(A) Study of the role of a putative metastasis suppressor gene nm23-H1 in mammary development and differentiation (see publication #5)

**Overexpression of nm23-H1 in human breast carcinoma cells leads to formation of basement membrane, production and apical secretion of sialomucins and growth arrest**

To determine the effect of nm23-H1 gene expression on the morphological differentiation of breast cells, MDA-MB-435 breast carcinoma clonal cell lines transfected with pCMVBamneo vectors (C-100 and C-103) or the same vector containing the full length nm23-H1 cDNA (H1-170 and H1-177) were cultured within an EHS matrix for 12 days. Immunohistochemical staining of type IV collagen and laminin was conducted on parental MDA-MB-435 cells, control transfectants, and nm23-H1 gene transfectants cultured within an EHS matrix. By day 6 of culture,  $87.8\% \pm 3.8\%$  of Nm23 protein-positive transfectants deposited type IV collagen; this percentage increased to  $97.1\% \pm 1.9\%$  by day 12 of culture. In contrast, none of the parental cells, control transfectants, or Nm23 protein-negative H1-170 transfectants expressed type IV collagen or laminin. These results indicated that overexpression of nm23-H1 in MDA-MB-435 breast carcinoma clonal cell lines led to formation of endogenous basement membrane as seen in normal breast epithelial cells HMT-3522. In addition to basement membrane synthesis,  $49.5\% \pm 4.5\%$  of Nm23 protein-positive transfectants expressed sialomucin (a mammary differentiation marker) compared with  $5.5\% \pm 1.5\%$  of control.

To determine whether differentiation induced by ectopic expression of nm23-H1 was linked to signaling in growth arrest, cell number per colony and thymidine labeling indices were measured for C-100, C-103, H1-170, and H1-177 cell lines grown within an EHS matrix for 12 days. Nm23 protein-negative colonies contained a mean of 27 cells, while Nm23 protein-positive colonies contained a mean of 8.2 cells. The percentage of spheres that were basement membrane-positive- $^3\text{H}$  thymidine negative rose from 7.6% on day of culture to 70.1% on day 12. A concurrent decrease in the percentage of basement membrane-positive- $^3\text{H}$  thymidine-positive spheres was observed from 80.2% on day 6 to 27.0% on day 12. These data suggested the hypothesis that basement membrane synthesis and secretion, an early event in the 3D matrigel culture system, may signal an inhibition of cell growth.

To further address the specific aim #3, we have used differential display to isolate novel breast tumor suppressors. In the 1996-1997 annual progress report, we have described characterization of a putative tumor suppressor gene AZ-1 isolated by comparing message levels of two phenotypically and functionally distinct cell lines [pre-malignant (S2) and tumor (T4-2) cells] of the HMT3522 breast cancer progression series. In accord with the differential expression patterns shown by RT-PCR differential display, AZ-1 was found to be highly expressed in S2 cells and the levels were significantly lowered in T4-2 cells by northern analysis (Figure 1a). The 4.4-kb message size of AZ1 was also observed in several nonmalignant breast cells including nonmalignant (S1), MCF10A, and primary luminal epithelial cells from mammaplasties (Figure 1b). In contrast, AZ-1 message was low or not detected in ten breast epithelial cell lines examined. Moreover, in comparison with the level in normal breast tissues AZ-1 message was low or absent in all four breast carcinoma biopsies (Figure

1b).

Sequence analysis of AZ-1 indicates that it encodes a protein size of 64 kD. Secondary structure prediction of the AZ-1 protein showed that it contains an extended coiled-coil domain at its C-terminus and an upstream repressor LexA-like HTH motif. BLAST (Basic Local Alignment Search Tool) search results indicated that the putative full-length 471 amino acid of AZ-1 coding sequence did not match any known gene. However, the coiled-coil domain of AZ-1 protein sequence showed limited homology with the  $\alpha$ -helical rod region of plakin family proteins including plectin, desmoplakin and envoplakin. In addition, the distal residues of AZ-1 coiled-coil region contain sequences similar to the intermediate filament (IF) signature LEF motif.

Based on the differential expression patterns shown by northern analyses, it is plausible that AZ-1 gene could encode a breast tumor suppressor. We have tested this hypothesis by transfecting AZ-1 into T4-2 cells and examined the functional and phenotypic changes of the AZ-1 stable transfectants.

(B) Functional analysis of the putative tumor suppressor gene AZ-1 in the breast tumor development (manuscript in preparation)

#### **AZ-1 gene is re-expressed in phenotypically-reverted tumor cells**

Since AZ-1 is homologous to myosin heavy chain and plakin family proteins that are known to be involved in organization of cytoskeletal architecture, we were interested in determining whether AZ-1 gene expression could be modulated by phenotypic alteration of tumor cells in a reversion system described previously. Briefly, when T4 cells were cultured in the presence of inhibitory  $\beta$ 1-integrin antibody in a 3-D assay system, they reverted morphologically to S1-like cells. They formed acini while re-assembled a basement membrane, re-organized cytoskeletal network, suppressed cyclin D1 and were growth arrested. Interestingly, AZ-1 gene was up regulated in the reverted T4 cells (analogous to p21) to a level reminiscent of that seen in the S1 cells. The recurrence of AZ-1 in normalized breast tumor cells could be linked to its putative function in cytoskeletal reorganization. However, further studies will need to confirm this hypothesis.

#### **Ectopically-expressed AZ-1 in human breast tumor cells (T4-2) leads to normalization of cell morphology, reduced invasiveness and anchorage-independent growth**

The functional roles of AZ-1 in breast tumor progression were examined by transfecting a retroviral expression construct (pAZ1-LXSN) containing the full-length AZ-1 cDNA into T4-2 cells. T4-2 cells stably transfected with pLXSN and pAZ1-LXSN constructs were grown in a three-dimensional reconstituted basement membrane (EHS) culture. Cells transfected with a control pLAPSN construct encoding an alkaline phosphatase were also analyzed. Similar to the untransfected T4-2 cells, at day 10 both pLXSN and pLAPSN transfected cells outgrew to form large, disorganized colonies (average colony size 175  $\mu$ m). In sharp contrast, pAZ1-LXSN transfected cells formed normal size spheroids (average colony size 40  $\mu$ m) with more organized cytoskeletal organization reminiscent to that of the nonmalignant (S1) cells (Figure 2). Tumor suppression function of AZ1 was further examined by a matrigel invasion assay. In comparison with vector transfected T4-2 cells, ectopically expressed

AZ1 drastically reduced the invasion potential of the breast tumor cells by 70-85%. The clonogenicity (also known as anchorage-independent growth) of pAZ1-LXSN transfected T4-2 cells determined by a soft agar assay was only 10-20% of the vector controls. These data suggested when assayed in an *in vitro* system ectopically-expressed AZ-1 reverted the tumor cells to a more normal-like morphology and a less tumorigenic phenotype.

The *in situ* tumor suppressor role of AZ-1 was also examined by injecting the AZ-1 stable transfectants into nude mice. The preliminary results are encouraging and repeated experiments are in progress and the final results will be reported in the manuscript in preparation.

## CONCLUSIONS

We have provided a complete list of the accomplishments that addressed the three proposed specific aims in this final report for the grant titled "Mechanisms of Abnormal Cell-Matrix Interactions in Human Breast Cancer." The initial phase (1994-1995 and the early part of 1995-1996) of the research efforts were conducted under the supervision of Drs. Mina Bissell and Anthony Howlett, the former principal investigator of the grant. During this period, the investigation was focused on understanding the aberrant cell-extracellular matrix (ECM) interactions in breast tumor cells. One of the major findings was the functional and phenotypic reversion of the breast tumor cells cultured in a three-dimensional system by an inhibitory anti- $\beta$ 1 integrin antibody (AIIB2). Further studies are underway to understand the molecular mechanisms involved and to explore the clinical implication of the  $\beta$ 1 integrin reversion system.

The second phase of the research was conducted by Dr. Huei-Mei Chen, the designated principal investigator of the grant. One of the exciting findings under Dr. Chen's supervision is the isolation of a putative tumor suppressor gene AZ-1. AZ-1 protein has a coiled-coil domain that shows homology to the dimerization domain of the plakin family proteins including desmoplakin. One of the interesting functions of AZ-1 revealed by the *in vitro* matrigel invasion assay was the demonstrated "invasion suppression" capability. The tumor suppressor function of AZ-1 was also examined in an *in vivo* system and the preliminary results were encouraging. Recent attempts on the biochemical characterization of AZ-1 protein by immunoprecipitation with anti-AZ-1 antibody showed AZ-1 may be involved in the intricate cell adhesion network and further studies are underway to extend our understanding of molecular mechanisms underlying the functions of this newly-discovered novel tumor suppressor candidate gene.

Research findings reported here outline only the beginning of the committed long-term efforts set forth to uncover the mysteries involved in the breast tumor progression. We will continue to unravel breast tumor relevant genes by differential display protocol employed in this study and to explore functions of these genes in the established breast cell culture and *in vivo* systems.

## REFERENCES

1. Gould,MN, 1993. Seminar in Cancer Biol. 4:161.
2. Wellings SR et al., 1975. J. Natl. Cancer Inst. 55:231.
3. Callahan, R et al., 1989. Breast Cancer Res. Treat. 13:191.
4. Holt, JT et al., 1993. Cancer Surv. 18:115.
5. Stoker, AW et al., 1990. Curr. Opin. Cell Biol. 2, 864.
6. Adams, UC and Watt, FM, 1993. Development 117,1183.
7. Hay,ED. 1993. Curr. Opin. Cell Biol. 5, 1029.
8. Streuli, CH et al., 1991. J. Cell Biol. 115, 1383.
9. Petersen, OW et al., 1992. PNAS USA 89, 9064.
10. Howlett, AR and Bissell, MJ, 1993. Epithelial Cell biol. 2, 79.
11. Chiquet-Ehrishmann, R et al., 1986. Cell 47,131.
12. Haslam, SZ, 1991. In Regulatory Mechanisms in Breast Cancer (ed. M. Lippmann and R. Dickson), pp. 401-420. Hingham: Kluwer.
13. Roskelley, CD et al., 1993. Advances in Mol. And Cell Biol. Vol. 7 (ed. G. Heppner), pp.89-113. New York:JAAAI Press.
14. Hynes, RO 1992. Cell 69, 11.
15. Damsky, CH and Werb, Z, 1992. Curr. Opin. Cell Biol. 4,772.
16. Juliano, RL and Haskill, S, 1993. J. Cell Sci. 108, 595.
17. Natali, PG et al., 1992. Br. J. Cancer 66, 318.
18. Zutter, MM et al., 1993. Am J. Pathol. 142, 1439.
19. Berdichevsky, F et al., 1994. Mol. Cell. Differ. 2, 255.
20. Gui, GPH et al., 1995. Surgery 117,102.
21. Howlett, AR et al., 1995. J. Cell Sci. 108,1945.
22. Rossen, K et al., 1994. Acta Dermato-Venereologica. 74, 101.
23. Weaver, VM et al., 1995. Semin. Cancer Biol. 6, 175.
24. Briand, P et al., 1987. In Vitro Cell Dev.Biol. 23, 181.
25. Briand, P et al., 1996. Cancer Res. 56, 2039.
26. Nielsen, KV and Briand, P, 1989. Cancer Genet. Cytogenet. 39, 103.
27. Madsen, MW et al., 1992. Cancer Res. 52, 1210.
28. Liang, P and Pardee, AB, 1992. Science 257, 967.
29. Sager, R. et a., 1993. FASEB J. 964.
30. Ruhrberg C and Watt FM. 1997. Curr. Opin. in Genetics & Develop 7:392.
31. Green KJ, et al., 1990. J Biol Chem, 265:2603.
32. Green KJ, et al., 1992. Int J Biol Macromol 14:145.
33. Ruhrberg C, et al., 1996. J Cell Biol 134:715. 37. McLean WH, et al. 1996, Genes Dev 10:1724.
34. Sawamura D, Li K, Chu ML, Uitto J. 1991. J Biol Chem 266:17784.
35. Virata Ml, Wagner RM, Parry DA, Green KJ., 1992. Proc Natl Acad Sci USA 89:544.
36. Wiche G, et al., 1991. J Cell Biol 114:93.
37. McLean WH, et al. 1996. Genes Dev. 10:1724.
38. Garrod DR. Desmosomes and Cancer. Cancer Survey 1995, vol.24, Cell Adhesion and Cancer, pp. 97.
39. Green KJ, Strappenbeck TS, Noguchi S et al.1991. Exp Cell Res. 193:134.
40. Stappenbeck TS, Bornslaeger EA, Corcoran CM, et al., 1993. J Cell Biol. 123:691.

## APPENDICES

### 1. LIST OF PUBLICATIONS AND MEETING ABSTRACT

#### Publications:

1. Close MJ, Howlett AR, Roskelley CD, Desprez PY, Bailey N, Rowning B, Teng CT, Stampfer MR, and Yaswen P. Lactoferrin expression in mammary epithelial cells is mediated by changes in cell shape and actin cytoskeleton. *J. Cell Sci.* 1997,110:2861-2871.
2. Howlett AR, Bailey N, Damsky C, Petersen OW, and Bissell MJ. Cellular growth and survival are mediated by  $\beta 1$  integrins in normal human breast epithelium but not in breast carcinoma. *J. Cell Sci.* 1995, 108:1945-1957.
3. Weaver VM, Howlett AR, Langton-Webster B, Petersen OW, and Bissell MJ. The development of a functionally relevant cell culture model of progressive human breast cancer. *Seminars in Cancer Biology*, 1995, 6(3):175-184.
4. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, and Bissell MJ. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* 1997, 137:231-245.
5. Howlett AR, Petersen OW, Streeg PS, and Bissell MJ. A novel function for the nm23-H1 gene: overexpression in human breast carcinoma cells leads to the formation of basement membrane and growth arrest. *J Natl Cancer Inst* 1994, 86:1838-1844.
6. Chen HM, Weaver VM, Petersen OW, and Bissell MJ. Extracellular matrix (ECM) as a central regulator of function, growth and programmed cell death in breast cells of both mice and men: implications for therapy. 1996, *The Pezcoller Foundation Journal* 3, p.7-11.

#### Manuscript in preparation:

Chen HM, Petersen OW, S. Mian and Bissell MJ. 1998. A novel putative tumor suppressor AZ- 1 and its plausible role in cytoskeletal reorganization.

#### Meeting Abstracts:

1. Weaver VM, Clark S, Petersen OW, and Bissell MJ. 1995. Refractoriness to  $\beta 1$ -integrin antibody-induced apoptosis precedes malignant transformation in HMT-3522 mammary epithelial cells: a culture model of progressive human breast cancer. Abstract submitted for American Society for Cell Biology meeting, Dec. 10-14. Washington DC.

2. Chen HM, Weaver VM, Wang F, Petersen OW, and Bissell MJ. 1997. A novel gene lost when human breast cells become malignant could serve as a progression marker. Abstract submitted for DOD Era of Hope meeting. Oct.31- Nov.4, Washington DC.

3. Chen HM, Petersen OW, and Bissell MJ. 1997. A novel gene lost when human breast cells become malignant is reexpressed in reverted cells. Abstract submitted for American Society for Cell Biology meeting meeting, Dec.13-17, Washington DC.

## 2. LIST OF PERSONNEL

Weaver VM (postdoctoral fellow)

Clark S (Research Technician)

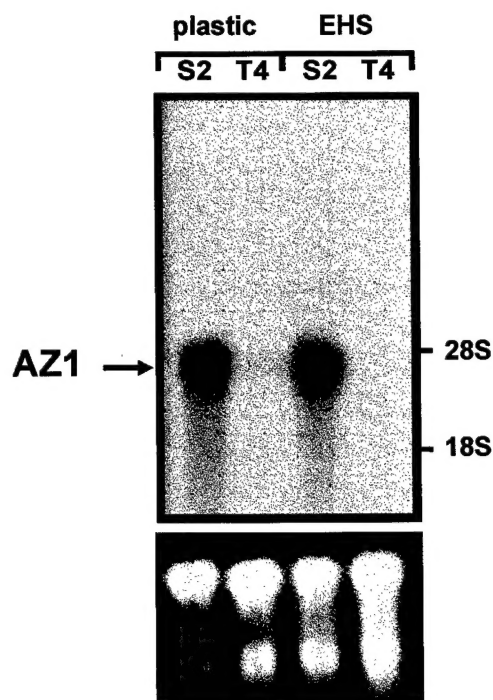
Zhou JM (Senior Research Associate)

Yue XM (Research Associate)

Chen HM (Principal Investigator)

Figure 1. Downregulation of AZ1 gene  
in breast tumor cell lines

a.



b.

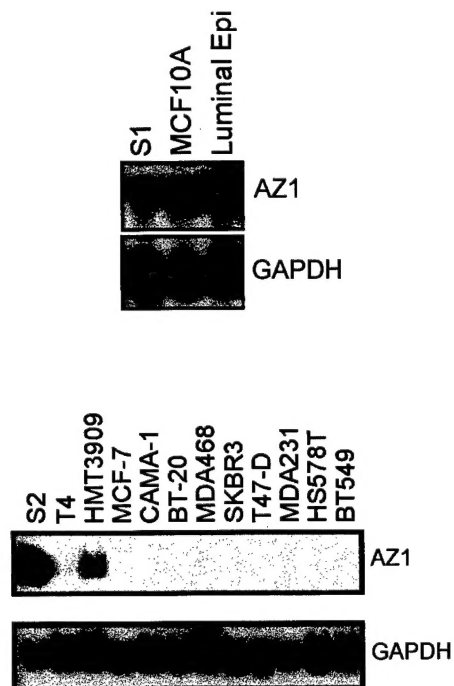


Figure 1. Downregulation of AZ1 message in breast carcinoma cells. Northern blot analysis was performed on RNAs from a) S2 (pre-malignant) and T4-2 (tumor) cells, grown on tissue culture plastic or in a three-dimensional (3-D) basement membrane assay (EHS); b) Nonmalignant (S1 and MCF10A) and malignant breast epithelial cell lines grown on tissue culture plastic. AZ1 message (arrow) was 4.4 kb. The bottom panel of Figure 2a is ethidium bromide stain of the RNA gel. While AZ1 message was abundant in nonmalignant and pre-malignant cells, it was either expressed at a low level or absent in the breast tumor cell lines examined. GAPDH probe was used as a control for the amount of RNA loaded.

**Figure 2. Ectopically-expressed AZ1 reverts breast tumor cell morphology**

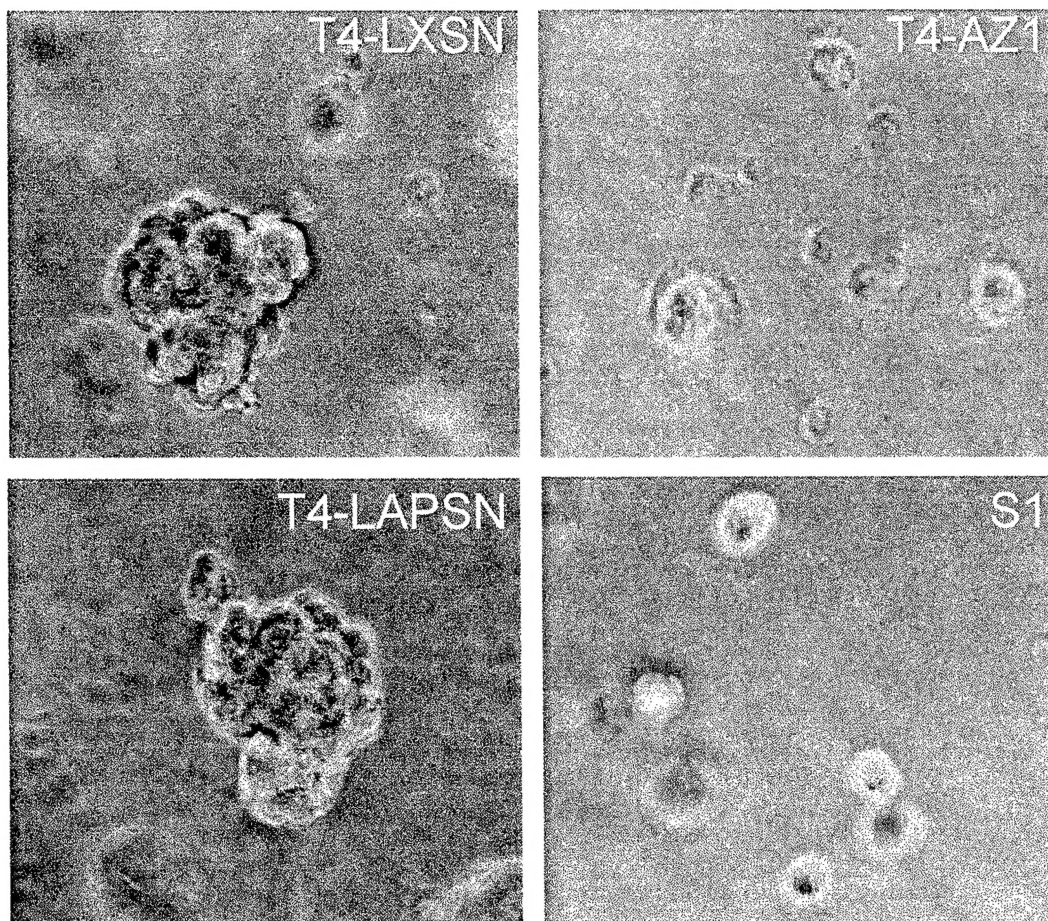


Figure 2. Reversion of tumor cell morphology in AZ1-transfected malignant breast epithelial cells. T4-2 cells were stably-transfected with LXSN (vector only), AZ1-LXSN (full-length AZ1) and AP-LXSN (AP, alkaline phosphate) constructs. The cell morphology of the transfectants and nonmalignant S1 cells were assayed in a three-dimensional (3-D) basement membrane culture. The AZ1-transfectants showed a normal size colony and a more organized spheroid structure as compared with that of vector and AP controls.